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Micelles of zinc protoporphyrin conjugated to *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer for imaging and light-induced antitumor effects in vivo

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ABSTRACT

We synthesized *N*-(2-hydroxypropyl)methacrylamide polymer conjugated with zinc protoporphyrin (HPMA-ZnPP) and evaluated its application for tumor detection by imaging and treatment by light exposure using in mouse sarcoma model. To characterize HPMA-ZnPP micelle, we measured its micellar size, surface charge, stability, photochemical, biochemical properties and tissue distribution. In vivo anti-tumor effect and fluorescence imaging were carried out to validate the tumor selective accumulation and therapeutic effect by inducing singlet oxygen by light exposure. HPMA-ZnPP was highly water soluble and formed micelles spontaneously having hydrophobic clustered head group of ZnPP, in aqueous solution, with a hydrodynamic diameter of 82.8 ± 41.8 nm and zeta-potential of $+1.12$ mV. HPMA-ZnPP had a long plasma half-life and effectively and selectively accumulated in tumors. Although HPMA-ZnPP alone had no toxicity in S-180 tumor-bearing mice, light-irradiation significantly suppressed tumor growth in vivo, similar to the cytotoxicity to HeLa cells in vitro upon endoscopic light-irradiation. HPMA-ZnPP can visualize tumors by fluorescence after i.v. injection, which suggests that this micelle may be useful for both tumor imaging and therapy. Here we describe preparation of a new fluorescence nanoprobe that is useful for simultaneous tumor imaging and treatment, and application to fluorescence endoscopy is now at visible distance.

1. Introduction

Photodynamic therapy (PDT) employs a photosensitizer and cytotoxic light-induced singlet oxygen ($^1O_2$) generation. $^1O_2$ generation damages DNA, RNA, proteins and lipids, which leads to cell death. Porphyrin derivatives usually generate cytotoxic $^1O_2$ after light irradiation that corresponds to the absorption wavelength of porphyrin derivatives [1–3]. Laserphyrin® and Photofrin® and others are well known porphyrin derivatives that are approved for limited use in conventional clinical PDT for early-stage lung (bronchogenic) or superficial cancer accessible to exciting light (laser irradiation at 630 nm) [4,5]. However, small molecular photosensitizers are expected to be distributed throughout the body including skin and other organs, and most have limited tumor selectivity or tumor-imaging capacity. Thus, they would cause cutaneous hyper-photosensitivity as the major adverse effect, which limits therapeutic success.

To solve this problem, one can utilize macromolecular photosensitizers, which have much longer half-lives in circulation and gradually and selectively accumulate in tumor tissues because of the EPR (enhanced permeability and retention) effect, accompanying much less accumulation in normal tissue [6–11]. Our group previously reported that biocompatible macromolecules (MW >40 kDa) showed the EPR effect and accumulated selectively in tumors [6,12,13]. For the EPR effect to operate, the macromolecular surface charge is as important a determinant as is molecular size; a neutral to slightly negative charge and MW of >40 kDa are preferable for tumor targeting [6,12,14]. In this study, we utilized a conjugate of zinc protoporphyrin (ZnPP) and 12-kDa *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer, which has a neutral charge and is highly biocompatible. The conjugate behaved as a large macromolecule (apparent MW is 198-kDa), as do many polymer conjugates of low-molecular-weight micellar drugs that show preferential tumor accumulation [15–18].

Light-irradiated (at 420 nm, absorption max of ZnPP) ZnPP effectively generates $^1O_2$ and thereby exhibits potent cytotoxicity [18,19]. ZnPP is also a potent inhibitor of heme oxygenase-1 (HO-1), or HSP-32, which is a survival factor. HO-1 is highly upregulated in many cancer tissues in vivo and confers an antioxidative function to cells. Therefore, inhibition of HO-1 by ZnPP makes tumor cells more vulnerable to oxystress, the result being selective tumor regression. Most of normal cells are not affected because HO-1 in normal cells is expressed only at low level and insignificant. However, ZnPP is highly hydrophobic and soluble only in alkaline solutions or organic solvents. This

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insolubility of ZnPP in physiological aqueous solution hampers its therapeutic application. To overcome this obstacle, we developed water-soluble ZnPP micelles: one is styrene maleic acid copolymer (SMA) micelles that encapsulate ZnPP and forms nanomicelles (SMA-ZnPP), the other is pegylated ZnPP (PEG-ZnPP) [18–22]. Both ZnPP micelles alone exhibited antitumor activity, and light irradiation greatly enhanced this activity [18]. Despite high tumor accumulation of PEG-ZnPP and significant antitumor activity, the maximum ZnPP loading in PEG-ZnPP is theoretically about 6% (wt/wt), so the intravenous (i.v.) dose of PEG-ZnPP may become several grams to achieve therapeutic concentrations. Although ZnPP loading of SMA-ZnPP can be increased to about 50%, SMA-ZnPP micelles tended to accumulate predominantly in the liver and spleen [23]. Therefore, we aimed to develop another type of ZnPP micelles with greater tumor targeting and adequate loading of ZnPP.

Here, we describe the synthesis of HPMA-ZnPP, which spontaneously formed micelles in aqueous solution. We examined its size distribution, spectroscopic property, micelle stability, generation of $^1O_2$, cellular uptake, tumor and tissue distribution and antitumor activity in vivo when used with xenon light-irradiation. Other important results concern simultaneous in vivo fluorescence imaging of the whole animal from outside, and the therapeutic effect of the polymer-photosensitizer conjugate.

2. Materials and methods

2.1. Materials

Male ddY mice were purchased from Kyudo Co., Ltd, Saga, Japan. Protoporphyrin IX, zinc acetate, triethylamine, dimethylaminopyridine, diethylether, Tween 20 and egg lecithin of reagent grade were purchased from Wako Pure Chemical, Osaka, Japan. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Dojindo Chemical Laboratory, Kumamoto, Japan. 2,2,6,6-Tetramethyl-4-piperidone (4-oxo-TEMP) was purchased from Tokyo Chemical Industry, Tokyo, Japan. The HPMA polymer (mean MW ~12 kDa) we used contains one free amino group at the end, and was prepared at the Institute of Macromolecular Chemistry, Prague, Czech Republic.

2.2. Synthesis of HPMA-ZnPP

Scheme 1 shows the synthesis of HPMA-ZnPP conjugate, in which conjugation of carboxyl group of free ZnPP with either hydroxyl group or amino group of HPMA (mean MW 12 kDa) was carried out to form ester and amide bonds, respectively. In brief, 570 mg of HPMA as Scheme 1 and 281 mg of ZnPP were mixed in 50 ml of DMSO at 50 °C and reacted by addition of 1.0 g of triethylamine, 1.2 g of dimethylaminopyridine and 1.9 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as a catalyst for 12 h at 50 °C in the dark. After the reaction, HPMA-ZnPP conjugates were precipitated by addition of diethylether (200 ml), and reaction catalyst in the supernatant was removed by centrifugation. The conjugates were washed three times with diethylether to remove the reaction catalyst and DMSO. HPMA-ZnPP was purified via gel permeation chromatography (Bio-Beads SX-1, BioRad, Hercules, CA) using dimethylformamide (DMF) as elute. Peak fraction of elutes was ultrafiltrated with membrane filter with a cutoff molecular size of 100 kDa, to remove decomposed or unreacted small molecules and to replace the DMF to distilled water. Fluffy powder (635 mg) was obtained by lyophilization.

2.3. Gel permeation column chromatography

Analytical gel permeation column chromatography of HPMA-ZnPP was performed with Bio-Beads SX-1 using a column ($\varphi = 2.5$ cm, $L = 60$ cm) and eluted with DMF at a flow rate of 0.1 ml/min. 1.5 ml fractions of elutes were measured at absorbance at 422 nm, which corresponded to ZnPP absorbance.

2.4. Fluorescence spectroscopy and fluorescence polarization

HPMA-ZnPP at 10 μg/ml was dissolved in PBS containing Tween 20 (0.0005–0.5%) or urea (1–9 M), and fluorescence spectra were measured with a fluorescence spectrophotometer (FP-6600; JASCO, Tokyo). HPMA-ZnPP (2.5 μg/ml) or free ZnPP (0.5 μg/ml) was dissolved in DMF, and sample solutions were then excited at 420 nm by a fixed polarized light; fluorescence emission at 590 nm was recorded at parallel (0°) and perpendicular (90°) angles of the secondary polarizer, which was equipped in a Model FP-6600 fluorescence spectrophotometer. The fluorescence polarization value (P value) was calculated by using the equation $P = (I_{//} - I_{\perp})/(I_{//} + I_{\perp})$, where $I_{//}$ = fluorescence intensity of the parallel component and $I_{\perp}$ = fluorescence intensity of the perpendicular component. The fluorescent polarization value is proportional to the molecular size of the fluorescent probe [24].

2.5. High performance liquid chromatography (HPLC)

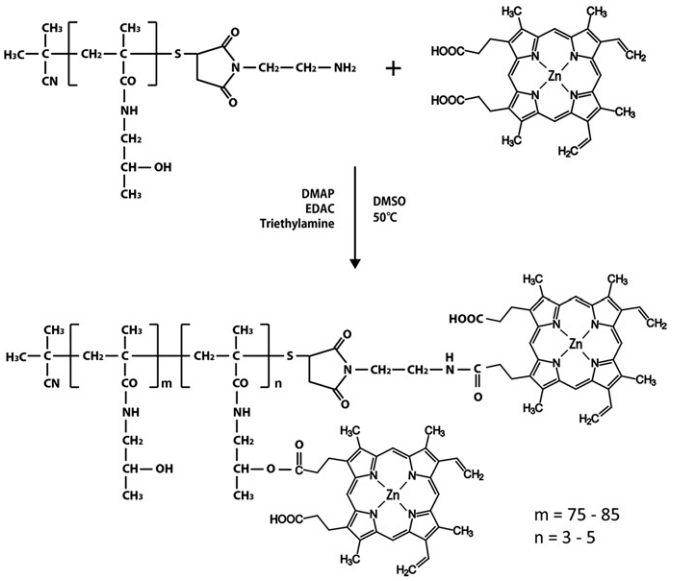
Cleavage of ester bond of this conjugate HPMA-ZnPP was analyzed by using HPLC (Prominence, Shimadzu, Kyoto, Japan) with the multimode size exclusion column GF-310 HQ (300×7.5 mm) with photodiode array detection at 422 nm, which was eluted with a mixture of 30% DMSO and 70% methanol containing 10 ppm trifluoroacetic acid at 1.0 ml/min.

2.6. Dynamic light scattering and zeta potential

HPMA-ZnPP or HPMA was dissolved in 0.01 M phosphate-buffered 0.15 M saline (PBS, pH 7.4) at 1 mg/ml and was filtered through a 0.2 μm filter attached to a syringe. The particle size and surface charge (zeta potential) were measured by light scattering (ELS-Z2; Otsuka Photal Electronics Co. Ltd., Osaka).

2.7. Transmission electron microscopy (TEM)

A drop of HPMA-ZnPP (0.1 mg/ml) was applied to a copper grid coated with carbon film and air-dried. The micelle image and size of



Scheme 1. HPMA-ZnPP synthesis. Chemical structures and conjugation pathway. ZnPP was conjugated to the secondary hydroxyl group and the terminal amino group of HPMA.